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Excessive S-Adenosyl-L-Methionine-Dependent Methylation Increases Levels of Methanol, Formaldehyde and Formic Acid in Rat Brain Striatal Homogenates: Possible role in S-adenosyl-L-methionine-induced Parkinson's disease-like disorders

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Abstract

Aims—Excessive methylation may be a precipitating factor for Parkinson's disease (PD) since S-adenosylmethionine (SAM), the endogenous methyl donor, induces PD-like changes when injected into the rat brain. The hydrolysis of the methyl ester bond of the methylated proteins produces methanol. Since methanol is oxidized into formaldehyde, and formaldehyde into formic acid in the body, we investigated the effects of SAM on the production of methanol, formaldehyde and formic acid in rat brain striatal homogenates and the toxicity of these products in PC12 cells.

Main methods—radio-enzymatic and colorimetric assays, cell viability, Western blot.

Key findings—SAM increased the formation of methanol, formaldehyde and formic acid in a concentration and time-dependent manner. Concentrations of [³H-methyl]-SAM at 0.17, 0.33, 0.67 and 1.34 nM produced 3.8, 8.0, 18.3 and 34.4 fmol/mg protein/h of [³H] methanol in rat striatal homogenates, respectively. SAM also significantly generated formaldehyde and formic acid in striatal homogenates. Formaldehyde was the most toxic metabolite to differentiated PC12 pheochromocytoma cells in cell culture studies, indicating that formaldehyde formed endogenously may contribute to neuronal damage in excessive methylation conditions. Subtoxic concentration of formaldehyde decreased the expression of tyrosine hydroxylase, the limiting factor in dopamine synthesis. Formaldehyde was more toxic to catecholaminergic PC12 cells than C6 glioma cells, indicating that neurons are more vulnerable to formaldehyde than glia cells.

Significance—We suggest that excessive carboxymethylation of proteins might be involved in the SAM-induced PD-like changes and in the aging process via the toxic effects of formaldehyde.

Keywords

Parkinson's disease; protein methylation; tyrosine-hydroxylase; PC12 cells

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Introduction

Parkinson's disease (PD) is a neurological disorder characterized by symptoms such as tremors, hypokinesia, rigidity and abnormal posture due to degeneration of dopaminergic neurons in the nigrostriatal pathway of the brain, resulting in dopamine depletion in the striatum (Hornykiewicz 1966; Dauer and Przedborski 2003). The etiology of PD is unknown, but a number of causative factors have been proposed, including mitochondrial impairments, oxidative stress (Mizuno et al. 1987; Halliwell 1992), environmental neurotoxins such as paraquat and manganese, and endogenous toxic substances that have a similar structure to 1-methyl-4-phenyl-tetrahydropyridine (MPTP), such as methyl β -carboline and N-methylsalsolinol. (Collins et al. 1992; Maruyama et al. 1997; Naoi et al. 2000). Excessive methylation might be involved in the pathogenesis of some cases of PD because injection of S-adenosyl methionine (SAM) into the rat brain induces PD-like behavioral, biochemical, and histological changes (Charlton and Way 1978; Crowell et al. 1993; Charlton and Mack 1994). Interestingly, several other proposed toxins underlying PD, such as methyl β -carboline and N-methyl salsolinol, are also methylated compounds. Moreover, higher methylation activity was found in PD patients (Aoyama et al. 2000), indicating the possible role of methylation in the pathogenesis of PD.

SAM is synthesized via catalytic activity of methionine adenosyltransferase, which transfers the adenosyl group of ATP to methionine. S-adenosylhomocysteine (SAH), formed after SAM transfers a methyl group to a methyl acceptor (Fig. 1), is then hydrolyzed to adenosine and homocysteine by SAH hydrolase. SAM is a universal endogenous methyl donor and is a limiting factor in various methylation reactions, including the methylation of proteins, phospholipids, DNA, RNA and other molecules including dopamine. Protein methylation is a post-translational modification and can be classified as either N-methylation or carboxymethylation (Roe 1943). Carboxymethylation involves the methylation of the –COOH group of amino acids in proteins, which is catalyzed by methyltransferases, resulting in the production of carboxyl methyl esters. Carboxyl methyl esters are unstable and readily hydrolyzed in neutral and basic pH conditions or by methylesterase to produce methanol (Gagnon et al. 1978; Wolf and Roth 1985). Methanol is oxidized into formaldehyde which, in turn, is oxidized into formic acid by alcohol dehydrogenase and aldehyde dehydrogenase. Although protein carboxymethyltransferase is widely distributed, the brain is one of the organs that exhibit the highest specific activities in the body (Diliberto and Axelrod 1976) and will be a major source for toxic byproducts of protein carboxyl-methylation. Protein carboxymethylation is involved in various physiological functions, such as chemotaxis (O'Dea et al. 1978) and secretion (Diliberto and Axelrod 1976).

The mechanism of methanol toxicity has been controversial, although the metabolism of methanol to formaldehyde and further to formic acid in the body was understood as early as 1943 (Roe 1943). After methanol is produced from the SAM-dependent protein carboxymethylation, it is metabolized by sequential oxidation to form formaldehyde and formic acid (Tephly and McMartin 1984; Eells et al. 1992). While methanol poisoning causes progressive visual impairment and blindness (Eells et al. 1992), methanol itself is considered relatively non-toxic. However, the metabolic products of methanol, such as formaldehyde and formic acid, are considered toxic. During the oxidative metabolism of methanol to formic acid, free radicals can be generated and may cause extensive damage to tissue (Datta and Namasivayam 2003). Formaldehyde induces DNA-protein crosslinks (Craft 1987) while formic acid induces metabolic acidosis in methanol poisoning (Clay et al. 1975; Walker 1975). The toxic effects of formic acid are caused by an inhibition of the cytochrome oxidase complex at the end of the respiratory chain in mitochondria (Nicholls 1976).

In the present study, the effects of SAM-induced protein carboxymethylation on the formation of methanol, formaldehyde and formic acid in rat brain striatal tissues were investigated. Cellular effects of the three metabolites- methanol, formaldehyde and formic acid-were also studied to further understand the possible role of these metabolites resulting from SAM-dependent protein carboxymethylation in SAM-induced PD-like changes using cell culture systems with catecholaminergic PC12 cells and C6 glioma cells.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 200-300 g were purchased from Harlan Laboratories (Indianapolis, IN) and maintained in a colony room under a 12-hr light and 12-hr dark cycle at constant temperature (22°C), with access to water and food *ad libitum*. The animals were acclimatized at least 5 days before being used for the experiments.

Chemicals

[³H-methyl]SAM (55.1 Ci/mmol of specific activity) was purchased from PerkinElmer (Waltham, MA). NaIO₄, Purpald reagent, formate dehydrogenase, nicotinamide adenine dinucleotide (NAD), p-iodonitrotetrazolium violet (INT), diaphorase, methanol, formaldehyde, formic acid and S-adenosyl-L-methionine (SAM) were obtained from Sigma-Aldrich (St. Louis, MO). The primary antibody for tyrosine hydroxylase (TH) raised in rabbits was from Chemicon-Millipore (Billerica, MA) and the secondary antibody against rabbit IgG raised in goats was from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of striatal homogenates

The rat brain striatal regions were dissected out after decapitation of the animals under chloral hydrate anesthesia (400 mg/kg). Striatal tissue was homogenized with tissue homogenizer model K-120 from Polysciences (Niles, IL) in phosphate buffer saline (PBS) solution (10 ml/g of tissue), consisting of 154 mM sodium chloride, 6.63 mM sodium phosphate, and 1.06 mM potassium phosphate, pH 7.4. The homogenates were centrifuged at 20,000 × g (J2-MC model, Beckman) for 20 min at 4°C. The supernatant was stored at -70°C until analysis.

Measurement of methanol

Methanol was measured as described previously with a minor modification of the previous method (Stock et al. 1984; Mark et al. 1993). In brief, the reaction medium, containing striatal homogenate (0.5-1.0 mg protein), [³H-methyl] SAM (0.17-1.34 nM) and PBS, in a final volume of 100 µl was incubated at 37 °C for 1 h. The reaction was terminated by the addition of 50 µl of 1 M NaOH and the opened tubes containing samples were then placed in 20-ml scintillation vials containing 7 ml scintillation fluid secured upright to prevent the contents from spilling. The vials were capped and warmed to 67°C for 1 h so that volatile [³H] methanol could evaporate into the scintillation fluid. Then the tubes were removed after cooling to room temperature, and the radioactivity in the vials was counted in the scintillation counter (LC 6500, Beckman).

Measurement of formaldehyde

Formaldehyde was measured as described previously (Quesenberry and Lee 1996) with a minor modification. In brief, aliquots of striatal homogenate (0.5-1.0 mg protein), containing 0-1 mM concentrations of SAM in a final volume of 400 µl were incubated at 37°C for the indicated times. One ml of 34 mM Purpald was added to the mixture and incubated for another 20 min at room temperature prior to the final addition of 1 ml 66 mM NaIO₄ for color development. Absorbance was measured at 550 nm in a spectrophotometer (Hitachi).

Measurement of formic acid

The method for the measurement of formic acid was based on the previously described method (Triebig and Schaller 1980). The mixture, containing brain striatal homogenate (0.5-1.0 mg protein), SAM, formate dehydrogenase, NAD^+ and PBS in a final volume of 1 ml, was incubated at 37°C for 1 h. The reaction was terminated by the addition of 1 ml of 1mM p-iodonitrotetrazolium violet (INT) and 100 μl diaphorase. Formic acid was cleaved to carbon dioxide and water by formate dehydrogenase, whereby NAD^+ was reduced to NADH, which reacted with INT in the presence of NAD-diaphorase. Incubation was carried out overnight at room temperature for color development. The color thus produced was determined at 500 nm (Ogata and Iwamoto 1990).

Cell culture

PC12 and C6 glioma cells were purchased from American Type Culture Collection (Rockville, MD). PC12 cells are catecholamine-secreting adrenal pheochromocytoma cell lines and widely used as a neuron-like model system to study toxicity mechanisms (Lee et al. 2002). PC12 and C6 cells, during their exponential growth phase, were maintained in a tissue culture flask in an atmosphere of 5% CO_2 /95% air in RPMI 1640 medium for PC12 and Kaighn's modification of Ham's F12 medium (F12K) with 2 mM L-glutamine for C6 cells, containing 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 250 ng/ml amphotericin B. PC12 cells (2.5×10^5 cells/ml) were treated with 100 $\mu\text{g}/\text{L}$ nerve growth factor (NGF) in collagen-coated plates for 5 days and the medium was changed every 3 days until the experiments were conducted.

Cell viability assay

Cell viability was measured by alamar blue assay using resazurin as a dye indicator. In brief, after PC12 or C6 cells (2.5×10^5 cells/ml) were treated in 96-well plates with methanol, formaldehyde or formic acid in DMEM/F12 media containing 17.5 mM glucose, resazurin dye was added to the culture media to test cell viability. In this assay, dye solution was added to the culture medium in a final concentration of 50 $\mu\text{g}/\text{ml}$ and incubated for 4-8 h at 37°C. After incubation, the fluorescence produced by the enzyme from the live cells was measured in a fluorometer (Model 7620, Cambridge Tech) with settings at 550/580 nm (excitation/emission).

Western blots

PC12 cells were differentiated with 100 ng/ml NGF for 5 days in poly-D-lysine-coated 6-well plates. Formaldehyde (0-100 μM) was added to the PC12 cell culture media for the indicated times and cells were collected into tubes. Cells were washed with PBS by centrifugation at $500 \times g$ for 2 min and placed in a RIPA lysis buffer (containing a protease inhibitor cocktail, 10 $\mu\text{g}/\text{ml}$) for 2 h on ice followed by the centrifugation at $10,000 \times g$ for 5 min at 4°C. The pellet was discarded and the supernatant was used for the experiment. After protein determination, 10 micrograms of proteins were solubilized in SDS sample buffer containing 2-mercaptoethanol and separated by 10% SDS-polyacrylamide gel electrophoresis. After the separated proteins on the gels were transferred to PVDF membranes electrophoretically, Western blotting was performed using rabbit anti-tyrosine hydroxylase (Chemicon-Millipore, dilution 1:5000) as a primary antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, dilution 1:3000) was used to visualize the bands. To quantify the amount of TH expression, β -actin (Santa Cruz Biotechnology) was also detected as a housekeeping gene.

Statistical analysis

Data were expressed as the mean $\pm$ the standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test was used to determine

statistical differences between groups. A probability of 0.05 or less was considered a significant difference.

Results

Effect of SAM on the formation of methanol, formaldehyde and formic acid in rat brain striatal homogenates

The method applied in the present study, the radio-enzymatic assay for the measurement of methanol levels, was sensitive enough to detect the femtomolar range of methanol produced by striatal homogenates. The results showed that SAM increased the formation of methanol significantly in a concentration-dependent manner (Fig. 2A). Incubation of striatal homogenate with 0.33, 0.67 and 1.34 nM of [³H-methyl]-SAM produced 8.0 ± 0.4 , 18.3 ± 3.8 and 34.4 ± 6.0 fmol/mg protein/h of [³H] methanol, respectively. Concentrations of SAM used were similar to endogenous levels which are nM range. The production of methanol from SAM was slow and time dependent and plateaued at 4 h after the reaction started (Fig. 2B).

For the measurement of formaldehyde produced by SAM-dependent methylation, striatal homogenate was incubated with various concentrations of SAM up to 1000 μ M, which are relevant to endogenous levels of formaldehyde (μ M range, Heck et al. 1985). The results showed that SAM significantly increased the formation of formaldehyde. Concentrations of SAM at 50, 100, 500 and 1000 μ M generated 7.8 ± 1.6 , 17.5 ± 2.2 , 38.2 ± 6.0 and 66.3 ± 8.2 pmol/mg protein/h of formaldehyde in striatal homogenates, respectively (Fig. 3A). The production of formaldehyde plateaued at 4 h incubation, which is similar to the time course for methanol production by SAM (Fig. 3B).

For formic acid measurement, concentrations of SAM ranging from 62.5 to 1000 μ M were used to determine whether the brain increases the production of formic acid in response to increased methylation. The results showed that concentrations of SAM at 250, 500 and 1000 μ M significantly increased the formation of formic acid from a control value of 1.5 ± 0.3 to 4.0 ± 0.6 , 4.2 ± 0.2 and 4.5 ± 0.4 pmol/mg protein/h, respectively (Fig. 4A). The formation of formic acid plateaued at 4 h (Fig. 4B).

Effects of methanol, formaldehyde and formic acid on the viability of PC12 pheochromocytoma and C6 glioma cells

Since methanol, formaldehyde and formic acid were increased as a result of SAM-dependent protein methylation, we explored if these metabolites have toxic effects in vivo using a cell culture system. As shown in Fig. 5A and 5B, formaldehyde was highly cytotoxic to PC12 cells while methanol and formic acid showed no significant toxic effect under the present experimental conditions with up to 1.25 mM concentrations for 24 h incubation. The incubation of PC12 cells for 24 h at less than 1 mM of formaldehyde caused almost complete cell death. Concentrations of formaldehyde at 0.15, 0.3 and 0.6 mM decreased cell viability to 53.6 ± 7 , 15.0 ± 7 , and $2.3 \pm 2\%$, respectively. Under the same conditions, methanol and formic acid were not significantly toxic (Fig. 5A). In Fig. 5B, the duration of 2, 4, 8 and 24 h of incubation in 0.8 mM of formaldehyde decreased PC12 viability to 81.8 ± 9 , 67.0 ± 8 , 49.0 ± 7 and $2.3 \pm 2\%$, respectively.

We also explored whether formaldehyde might have selective toxicity to catecholamine-producing PC12 cells compared to C6 glioma cells derived from rat glioma. The results showed that formaldehyde caused significantly higher toxic effects in PC12 cells than C6 cells, indicating that catecholaminergic neurons such as dopaminergic neurons may be more vulnerable to formaldehyde (Fig. 6A,B,C). Formaldehyde at 3.15 mM reduced viability of PC12 cells to $55.5 \pm 4\%$ in 2 h incubation, while it had no significant effect on the viability of

C6 glioma cells. Formaldehyde at 12.5 mM for 2 h incubation reduced viability of PC12 cells to $2 \pm 7\%$, while it reduced viability in C6 glioma to $73.7 \pm 8\%$ (Fig. 6A). As shown with PC12 cells, the duration of exposure to formaldehyde also played a critical role in the toxicity for C6 cells (Fig. 6, A,B,C). For example, concentration at 6.3 mM has no significant effect on the C6 glioma cells during 2 h of exposure (Fig. 6A), but the same concentration for 8 h exposure reduced cell viability to $23.5 \pm 2\%$ (Fig. 6B). A much lower concentration, 0.6 mM ($\sim 10\times$ less) for 24 h exposure, reduced cell viability to $41.2 \pm 3\%$ (Fig. 6C).

Effects of formaldehyde on TH expression in PC12 cells

PC12 cells (1×10^6 cells/ml) were incubated with various concentrations of formaldehyde for 24 h after being differentiated with nerve growth factor (NGF, 100 ng/ml) for 5 days. Since the formation of formaldehyde was increased by SAM-dependent methylation, it was determined if formaldehyde affected TH expression levels. As shown in Fig. 7, formaldehyde significantly decreased expression of TH in PC12 cells.

Discussion

The results from the present study revealed that methanol, formaldehyde and formic acid are produced following incubation of rat striatal homogenates with S-adenosylmethionine (SAM). The formation of all three compounds reached maximum at 4 h incubation. Among the three metabolites, formaldehyde was the most toxic to PC12 cells and decreased TH expression, which is a rate-limiting factor in dopamine synthesis. These results indicate that excessive protein carboxymethylation might increase the amount of these toxic substances and may be responsible, at least in part, for neuronal damage observed in SAM-treated rats (Charlton and Mack 1994).

The formation of these metabolites is likely to be initiated by the process of carboxymethylation of proteins in which carboxymethylase catalyzes the transfer of the methyl group from SAM to the carboxyl terminus of proteins to produce a protein methyl ester. The ester is readily hydrolyzed to produce methanol (Diliberto and Axelrod 1976) and formaldehyde, derived from methanol, is metabolized to formic acid (Eells 1981; Treichel et al. 2003). Protein carboxymethylation is an ongoing process in the brain and increases during the aging process (Sellinger et al. 1988; Matsubara et al. 2002), implicating that the formation of these metabolites is a continuing event in the brain and other organs. The toxicities of methanol (Mellerick and Liu 2004), formaldehyde (Mellerick and Liu 2004; Hansen et al. 2005; Harris et al. 2004) and formic acid (Liesivuori et al. 1987) are well documented. Methanol has powerful solvent properties and is a precipitator of proteins. Formaldehyde is a cross-linker between protein and nucleic acids (Craft et al. 1987) and formic acid is a mitochondria inhibitor (Sharpe et al. 1982). Therefore, the production of these metabolites in the brain ought to display notable neurobiological effects. Although their toxic effects may be tempered by their low concentrations, during events of excessive methylation, levels of these metabolites will be increased and will cause notable toxic effects that may help to explain some of the changes observed following the administration of SAM that drives methylation and causes Parkinson's disease-like changes (Charlton and Mack 1994; Lamango et al. 2000).

The effects of protein methylation metabolites on the viability of neuronal-like cells may be indicative of their potential role in other biological processes. Since methylation is a normal process that increases during aging, these metabolites may also play a role in the aging process. In this context, the manner of toxicity is relevant as the interaction of formaldehyde with proteins is covalent, stable and irreversible. Therefore, at low concentrations and during its early actions, formaldehyde will cause imperceptible changes that will accumulate over time.

The physiological concentration of SAM in human brain is 1-2 nmol/ mg protein (Morrison et al., 1996) and 60 nM in cerebral spinal fluid in humans (Bottiglieri et al., 1990). In the present study, concentrations of SAM in the nM range were used to measure methanol, while the μ M range of SAM was used to measure formaldehyde and formic acid. It is possible that excessive methylation condition may lead the SAM level to become abnormally high in local compartmental areas of brain regions. Moreover, the concentration of formaldehyde in human blood is 2.6 μ g/g of blood ($\sim$ 50-100 μ M, Heck et al. 1985) and 0.097 μ mol/g tissue in the rat brain tissue (Heck et al. 1982). This indicates that formaldehyde levels in normal tissues are relatively high and thus, abnormal biochemical conditions, such as SAM-dependent excessive methylation, high intake of aspartame (an artificial sweetener), which produces methanol and formaldehyde (Oyama et al., 2002), or lack of GST-detoxifying mechanisms (Harris et al. 2004), could induce accumulation of formaldehyde and damage tissues.

Methylation may play a role in L-dopa-induced side effects. Methylation-related L-dopa metabolites such as 3-OMD and homocysteine are well known to cause toxicity (Muller et al. 2004; Lee et al. 2008). Long-term treatment with L-dopa, which may increase methylation activity due to the burden of L-dopa-metabolism, may increase other toxic methylated products such as N-methylated TiQs and O-methylated products such as protein carboxymethylation, which can produce methanol and formaldehyde. Due to complications associated with methylation in L-dopa therapy, it is imperative to pursue preventive treatment strategies. Concurrent intake of folic acid to metabolize excessive homocysteine and COMT inhibitors such as tolcapone and entacapone with L-dopa might be crucial in preventing the accumulation of toxic methylated products (Bonifacio et al. 2007).

The present studies that test the toxicity of these metabolites in neuronal-like PC12 and C6 glioma cells revealed that formaldehyde was the most potent and toxic among the three as shown for PC12 cells in Fig. 5A. The longer incubation of PC12 cells with formaldehyde dramatically increased its cytotoxicity as shown in Fig. 5B, suggesting that the duration of exposure is a critical determinant of formaldehyde's effect. The toxicity of formaldehyde suggests that the worsening symptoms and death seen following methanol intoxication may be due to formaldehyde. As protein carboxymethylation increases with age, the expression of formaldehyde toxicity is apparently a significant feature, indicating a role for methylation in the aging process. The neuronal-like PC12 cells were more sensitive to the toxic effects of formaldehyde than C6 glioma cells. Furthermore, the toxicity rate as a function of concentration is steeper for the PC12 cells, indicating that neuronal-like PC12 cells are more vulnerable to formaldehyde exposure compared to C6 glioma cells.

Protein carboxymethylation might have two different pathological effects in excessive methylation conditions. The immediate effects of protein methylation might be the methylation of specific proteins such as dopamine receptor proteins (Lee et al. 2004) and farnesyl methylation, which might be directly involved in SAM-induced tremors (Lamango and Charlton 2000). These effects occur shortly after SAM treatment. However, the long-term effects of protein methylation might be via the metabolites of carboxymethylation: methanol, formaldehyde and formic acid which in the present data reached maximum production 4 h after SAM treatment.

Taken together, the present study indicates that excessive protein carboxymethylation, increasing methanol, formaldehyde and formic acid, may cause neurotoxic effects via the potent metabolite formaldehyde, and may play a role in SAM-induced PD-like neuronal damage and the aging process.

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Fig. 1. Metabolic pathway of SAM-dependent protein methylation and formation of methanol, formaldehyde and formic acid.

Fig. 2.

(A) Effect of SAM on the production of methanol in striatal homogenates. Homogenates were incubated with various concentrations of $[^3\text{H-methyl}] \text{SAM}$ at 37°C for 1 h, followed by the procedure stated in Materials and Methods. Values are expressed as the mean $\pm$ SEM ($n=4$).

(B) Time-course study of SAM-induced methanol production in striatal homogenates. Homogenates were incubated with 0.33 nM $[^3\text{H-Methyl}] \text{SAM}$ for the times indicated, followed by the procedure stated in Materials and Methods. Values are expressed as the mean $\pm$ SEM ($n=4$).

Fig. 3.

(**A**) Effect of SAM on the production of formaldehyde in striatal homogenates. Homogenates were incubated with various concentrations of SAM at 37°C for 1 h, followed by the procedures described in the Materials and Methods. Values are expressed as the mean $\pm$ SEM (n=4). (**B**) Time-course study of SAM-induced formaldehyde production in striatal homogenates. Homogenates were incubated with 0.5 mM of SAM for the times indicated, followed by the procedure stated in the Materials and Methods. Values are expressed as the mean $\pm$ SEM (n=4). *indicates significant increase compared to the control (**P<0.01, ***P<0.001).

Fig. 4.

(**A**) Effect of SAM on the production of formic acid in striatal homogenates. Homogenates were incubated with various concentrations of SAM and formate dehydrogenase at 37°C for 1 h, followed by the procedure stated in Materials and Methods. Values are expressed as the mean $\pm$ SEM (n=4). (**B**) Time-course study of SAM-induced formic acid production in striatal homogenates. Homogenates were incubated with 0.5 mM of SAM and formate dehydrogenase for the times indicated, followed by the procedure stated in Materials and Methods. Values are expressed as the mean $\pm$ SEM (n=4). *indicates significant increase compared to the control (*P<0.05, ***P<0.001).

Fig. 5.

(A) Effects of methanol, formaldehyde and formic acid on cell viability in PC12 cells. Cells were incubated with various concentrations of methanol, formaldehyde and formic acid in 96-well plates for 24 h at 37°C. Cell viability was assessed by alamar blue assay using resazurin dye indicator as described in Methods. *indicates significant decrease compared to the control (** $P < 0.001$). (B) Concentration-dependent effects of formaldehyde on cell viability at various incubation times in PC12 cells. Cells were incubated with various concentrations of formaldehyde in 96-well plates for the times indicated at 37°C. Cell viability was assessed by alamar blue assay using resazurin dye indicator as described in Methods. *indicates significant decrease compared to the control (* $P < 0.05$, ** $P < 0.001$).

Fig. 6.

Comparison of formaldehyde-induced cytotoxicity in PC12 neuronal-like and C6 glioma cells. PC12 and C6 cells (2.5×10^5 cells/ml) were incubated with various concentrations of formaldehyde in 96-well plates for the times indicated at 37°C. Cell viability was assessed by alamar blue assay using resazurin dye indicator as described in Methods. *indicates significantly decreased compared to the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

A

Fig. 7.

Effect of formaldehyde on expression of TH in PC12 cells. **(A)** Cells were incubated with various concentrations of formaldehyde in 6-well plates (1×10^6 cells/ml) for 12 h at 37°C. After treatment, western blot analysis was performed. **(B)** PC12 cells were incubated with 25 μ M formaldehyde for the times indicated at 37°C. Quantitative analysis was performed and all data were normalized by corresponding β -actin band density. Values are expressed as the mean $\pm$ SEM (n=4). *indicates significant decrease compared to the control (*P<0.05).